Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

The specification beginning at p. 23, line 25, has been amended, as recommended by the United States Patent and Trademark Office ("PTO") at p. 3 of the office action, to incorporate the list of the essential HSV genes identified in Roizman B., "The Function of Herpes Simplex Virus Genes: A Primer for Genetic Engineering of Novel Vectors," *Proc. Natl. Acad. Sci.* 93:11307–11312 (1996) ("Roizman") (attached hereto as Exhibit A). Roizman, cited at p. 23, l. 30 of the specification and incorporated by reference at p. 9, ll. 9-10 of the specification, is a review article discussing the genomic structure and content of herpes simplex virus-1 (HSV-1). Table 1 of Roizman presents a list of HSV-1 genes and the function of the encoded gene products (Roizman at 11310, col. 1, 2nd full para.). Roizman explains that 45 of the 83 open reading frames ("ORFs") specifying diverse proteins are dispensable for viral replication (*id.*). The genes that cannot be deleted without ablating the capacity of the virus to replicate, *i.e.*, essential HSV genes, are denoted in column 3 of Table 1 of Roizman as genes that are not dispensable in cell culture (*id.*). The specification has been amended to incorporate a list of these essential HSV genes identified as such by Roizman. This amendment does not introduce new matter.

Likewise, the specification beginning at p. 34, l. 9 has been amended to incorporate the more detailed description of the method of helper virus-free amplicon packaging that is disclosed in Bowers et al., "Expression of vhs and VP16 During HSV-1 Helper Virus-Free Amplicon Packaging Enhances Titers," *Gene Therapy* 8(2):111-120 (2001) (attached hereto as Exhibit B), which is cited at p. 34, ll. 29-30 of the specification and incorporated by reference at p. 9, ll. 9-10 of the specification. This amendment does not introduce new matter.

Claims 28–31, 33, 34, 49, and 51 are currently pending. Claim 28 has been amended and claim 50 has been canceled. Support for the amendment to claim 28 is found in the specification at p. 23, ll. 2–8 and at p. 34, starting at l. 28 as amended above. Accordingly, no new matter has been introduced by way of this amendment.

The objection to the specification under 35 U.S.C. § 132(a) for new matter is respectfully traversed in view of the amendment to the specification to recite the "essential HSV genes." This objection should be withdrawn.

The rejection of claims 28–31, 33, 34, 49–51 under 35 U.S.C. § 112 (first paragraph) for lack of clarity is respectfully traversed in view of the amendment to the specification to recite the "essential HSV genes". This rejection should be withdrawn.

The rejection of claims 28–31, 33, 34, 49–51 under 35 U.S.C. § 112 (first paragraph) for containing new matter is respectfully traversed in view of the amendment to the specification to recite the "essential HSV genes". This rejection should be withdrawn.

The rejection of claims 28–31, 33, 34, and 49 under 35 U.S.C. § 112 (first paragraph) for lack of written description is respectfully traversed in view of the amendment to claim 28 above. Accordingly, this rejection should be withdrawn.

The rejection of claims 28–31, 33, 34, 49, and 50 under 35 U.S.C. § 103 for obviousness over U.S. Patent No. 7,014,855 to Schenk ("Schenk '855") in view of U.S. Patent No. 6,972,127 to Schenk ("Schenk '127"), U.S. Patent No. 6,946,135 to Schenk ("Schenk '135"), Stavropoulos and Strathdee, "An Enhanced Packaging System for Helper-Dependent Herpes Simplex Virus Vectors," *J Virol.* 72(9):7137–43 (1998) ("Stavropoulos"), Saeki et al., "Herpes Simplex Virus Type 1 DNA Amplified as Bacterial Artificial Chromosome in *Escherichia coli*: Rescue of Replication-Competent Virus Progeny and Packaging of Amplicon Vectors," *Human Gene Therapy* 9:2787–94 (1998) ("Saeki"), and Town et al., "Reduced Th1 and Enhanced Th2 Immunity After Immunization with Alzheimer's β-Amyloid$_{1-42}$," *J Neuroimmunology* 132:49–59 (2002) ("Town") is respectfully traversed.

Schenk '855 discloses methods of preventing or treating a disease characterized by amyloid deposition in a patient that involves inducing an immune response to a peptide component of the amyloid deposit. The methods of Schenk '855 involve both active induction of the immune response by administration of an amyloid-β (Aβ) immunogen and passive induction by administration of an antibody or antibody fragment specific for Aβ.

Schenk '127 similarly discloses methods of preventing or treating a disease characterized by amyloid deposition. Schenk '127 has apparently been cited for teaching that an immunogenic Aβ peptide can be delivered via a viral vaccine, and that HSV viral vectors are one of many suitable vectors for such delivery.

Schenk '135 also relates to methods of preventing or treating a disease characterized by amyloid deposition. Schenk '135 has apparently been cited for teaching that the Aβ peptide immunogen can be linked to a suitable carrier molecule to help elicit an immune response. Suitable carrier molecules disclosed by Schenk '135 include keyhole limpet hemocyanin and tetanus toxoid.

Stavropoulos describes the development of a second-generation packaging system for HSV amplicons. This system involves providing a single HSV bacterial artificial chromosome (BAC) containing the five overlapping HSV-1 cosmid clones encoding the complete wild-type HSV genome. The BAC also includes a *pac* cassette inserted at a BamHI site located within the U_L41 gene sequence, which encodes the virion host shutoff (vhs) protein. This insertion disrupts vhs protein expression.

Saeki describes another approach to HSV packaging that involves cloning the HSV-1 genome as an F plasmid based BAC in *E. coli*. Saeki demonstrates that the plasmid containing the HSV-1 genome, deleted for the *pac* signals, does not generate replication-competent progeny virus upon transfection into mammalian cells, but rather, was able to support the packaging of co-transfected amplicon DNA that contained a functional *pac* signal.

Town characterizes the Th1 and Th2 immune response following $A\beta_{1-42}$ and $A\beta_{1-40}$ peptide immunization in wildtype and an Alzheimer's disease mouse model. Town has been cited by the PTO for disclosing that an enhanced Th2 response and reduced Th1 response are observed following immune challenge with the $A\beta_{1-42}$ peptide.

The PTO asserts that it would have been obvious to produce the HSV vectors of Schenk for the delivery of a gene encoding Aβ and a gene encoding keyhole limpet hemocyanin using the helper-virus free methods of Stavropoulos and Saeki, and to deliver the resulting HSV amplicons to treat Alzheimer's disease. Applicants respectfully disagree.

Neither Stavropoulos nor Saeki teach a method of HSV amplicon particle production that involves *co-transfecting* a cell with (a) an amplicon plasmid comprising an HSV origin of replication, an HSV cleavage/packaging signal, and a heterologous transgene, (b) one or more vectors individually or collectively encoding all essential HSV genes but excluding all cleavage/packaging signals *and* (c) a nucleic acid encoding a virion host shut-off (vhs) protein where the nucleic acid encoding said vhs accessory protein is separate from the amplicon plasmid of (a) and the one or more vectors of (b) as recited in amended claim 28. The PTO

contends that Stavropoulos and Saeki each teach the delivery of amplicons containing a nucleic acid sequence encoding an accessory protein (office action at p. 8). However, the nucleic acid molecule referred to as an accessory protein (*i.e.*, the antibiotic resistance gene for ampicillin) is a component of the HSV amplicon. In contrast, the accessory vhs protein of the claimed invention is separate and distinct from the amplicon plasmid and the one or more vectors encoding all essential HSV genes. As described in the specification at p. 34, starting at l. 28 as amended above, the amplicon plasmid, the one or more vectors (*e.g.*, cosmid DNA vectors), and the nucleic acid encoding the accessory vhs protein are separate and distinct DNA fragments that are co-transfected into recipient cells (*e.g.*, BHK cells). Neither Stavropoulos nor Saeki alone or in combination with the other cited references teach or suggest a method that involves the co-transfection of a separate and distinct nucleic acid encoding a vhs accessory protein. In fact, Stavropoulos, by explicitly teaching the disruption of the vhs gene to prevent its delivery and expression during amplicon production, teaches away from the claimed invention. Accordingly, the rejection of claims 28–31, 33, 34, 49 and 50 for obviousness over the combination of Schenk '855, Schenk '127, Schenk '135, Stavropoulos, Saeki, and Town is improper and should be withdrawn.

The rejection of claims 28–31, 33, 34, 49 and 50 under 35 U.S.C. § 103 for obviousness over Schenk '855, Schenk '127, Schenk '135, Stavropoulos, Saeki, Town, and further in view of Whitley et al., "Herpes Simplex Viruses," *Clin Infect Dis* 26:541–55 (1998) ("Whitley") is respectfully traversed.

The PTO, acknowledging that neither Stavropoulos nor Saeki teach that a nucleic acid encoding vhs is explicitly included in their respective systems to produce HSV amplicons, has apparently cited Whitley for this reason. Applicants respectfully disagree.

Firstly, applicants submit that persons of skill in the art would not have considered combining the teachings of Whitley with Schenk '855, Schenk '127, Schenk '135, Stavropoulos, Saeki, and Town, because Whitley represents non-analogous art and, as such, one of ordinary skill would not have had any motivation to consider Whitley at the time the present invention was made.

In determining whether a reference constitutes "analogous art", the Federal Circuit has set forth a two-part test inquiring (1) whether the art is from the same problem solving field of endeavor, and (2) if the reference is not within the field of the inventor's

endeavor, whether the reference is still reasonably pertinent to the particular problem with which the inventor is involved. *See* In re Clay, 966 F.2d 656, 658-659, 23 USPQ2d 1058, 1060-1061 (Fed. Cir. 1992). This test was recently applied by the Federal Circuit in *In re Arnold G. Klein*, 98 USPQ2d 1991 (Fed. Cir. 2011), where the Court examined the sufficiency of references from different problem solving fields, *i.e.*, application of the second prong of the test. For references not within the field of the inventor's endeavor, the Federal Circuit held that it was improper to rely on references directed to solving different problems where no other reasoning is provided to justify reliance on the reference. *In re Arnold G. Klein*, 98 USPQ2d at 1995-96.

Whitley is a review article of herpes simplex viruses that discusses HSV structure and replication, HSV infection and clinical manifestations, and current strategies for the prevention and treatment of HSV infection. Whitley teaches that vhs, the product of the U_L41 gene, plays an important role in natural viral infections by inducing an RNA activity which degrades all mRNA.

The claimed invention relates to a method of treating a patient having a neurodegenerative disease that involves administering to the patient an HSV amplicon particle produced by a helper-virus free method. The helper-virus free method of amplicon production involves co-transfecting a cell with (a) an amplicon plasmid comprising a therapeutic protein, (b) one or more vectors that individually or collectively encode all essential HSV genes, and (c) a nucleic acid encoding a vhs accessory protein.

Thus, the problem-solving area of the present invention is treating a patient having a neurodegenerative disease using HSV amplicons produced using a helper-virus free method as described in the preceding paragraph. In contrast, the problem-solving area of Whitley concerns prevention and treatment of HSV infections. Whitley's discussion of vhs function is entirely unrelated to its effect on amplicon production or titer in a helper-virus free system of amplicon production. As such, Whitley is not at all pertinent to the invention as claimed, and the PTO has failed to establish any facts to support the pertinence thereof.

Because Whitley is not at all related to the problem-solving area of the claimed invention and the PTO has failed to establish any pertinence between Whitley and the claimed invention, Whitely constitutes non-analogous art and cannot be cited against the claimed subject matter

Even if Whitely is analogous art, which applicants do not agree that it is, Whitely fails to teach a helper virus-free method of amplicon production that involves co-transfecting a cell with (a) an amplicon plasmid comprising heterologous transgene, (b) one or more vectors individually or collectively encoding all essential HSV genes and (c) a nucleic acid encoding a virion host shut-off (vhs) protein where the nucleic acid encoding said vhs accessory protein is separate from the amplicon plasmid of (a) and the one or more vectors of (b) as recited in amended claim 28. Therefore, Whitely does not overcome the above-noted deficiency of the combination of Schenk '855, Schenk '127, Schenk '135, Stavropoulos, Saeki, and Town.

For all of these reasons, the rejection of claims 28–31, 33, 34, 49 and 50 for obviousness over Schenk '855, Schenk '127, Schenk '135, Stavropoulos, Saeki, Town, and Whitley is improper and should be withdrawn.

In view of all of the foregoing, applicant submits that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: August 23, 2011

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Exhibit A: Roizman B., "The Function of Herpes Simplex Virus Genes: A Primer for Genetic Engineering of Novel Vectors," *Proc. Natl. Acad. Sci.* 93:11307-11312 (1996)

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11307–11312, October 1996
Colloquium Paper

This paper was presented at a colloquium entitled "Genetic Engineering of Viruses and of Virus Vectors," organized by Bernard Roizman and Peter Palese (Co-chairs), held June 9–11, 1996, at the National Academy of Sciences in Irvine, CA.

The function of herpes simplex virus genes: A primer for genetic engineering of novel vectors

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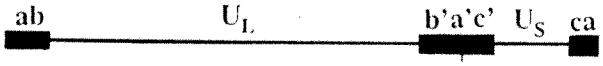
ABSTRACT **Herpes simplex virus vectors are being developed for delivery and expression of human genes to the central nervous system, selective destruction of cancer cells, and as carriers for genes encoding antigens that induce protective immunity against infectious agents. Vectors constructed to meet these objectives must differ from wild-type virus with respect to host range, reactivation from latency, and expression of viral genes. The vectors currently being developed are (*i*) helper free amplicons, (*ii*) replication defective viruses, and (*iii*) genetically engineered replication competent viruses with restricted host range. Whereas the former two types of vectors require stable, continuous cell lines expressing viral genes for their replication, the replication competent viruses will replicate on approved primary human cell strains.**

Herpes simplex viruses (HSV) and particularly HSV-1 are potential vectors for several applications in human health. These include (*i*) delivery and expression of human genes to central nervous system (CNS) cells, (*ii*) selective destruction of cancer cells, and (*iii*) prophylaxis against infections with HSV and other infectious agents. The properties of wild-type virus are fundamentally antithetical to such applications. HSV-1 is highly destructive to infected cells. In addition, HSV-1 is generally spread by contact of the tissues containing virus of one individual with mucous membranes of an uninfected individual. The virus multiplies at the portal of entry, infects sensory nerve endings innervating the site of multiplication, and is transported retrograde to the nucleus of sensory neurons. The sequence of events beyond this point are less well known. In experimental animal systems, the virus multiplies in some neurons but establishes a latent state in others. In a fraction of those infected, the virus periodically reactivates from latent state. In these neurons the newly replicated virus is transported anterograde, usually to a site at or near the portal of entry into the body, where it may cause a localized lesion. In immunosuppressed individuals, the lesions caused both by initial infection and recrudescences tend to be more extensive and persist longer than in immunocompetent individuals (reviewed in refs. 1 and 2). To serve as vectors, the viral genotype must be extensively altered to fit the objective of the vector. For example, to deliver and express human genes in the CNS, the desirable properties of HSV are its ability to establish latent infections and the huge coding capacity of the viral genome. The undesirable property is the capacity of the virus to commit the cell to destruction very early in infection. For selective destruction of cancer cells, the desirable property is the capacity of the virus to destroy cells. The undesirable properties are the wide host range of the wild-type virus and the capacity of the virus to reactivate from latent state. Each application therefore requires a different kind of vector and, in principle, different kinds of genetic engineering. The purpose of this report is to summarize our knowledge of the molecular biology of HSV-1 relevant to experimental design of viral vectors.



Genome Domains	Open Reading Frames	No.
L Component		
ab, b'a'	α0, $\gamma_1$34.5, ORF-P, O	8
U_L	U_L1-56, 8.5, 9.5, 10.5 12.5, 20.5, 26.5, 27.5 43.5, 49.5	65
S Component		
a'c', ca	α4	2
U_S	U_S1-12, 1.5, 8.5	14
	Total	89
	Total single copy	84

FIG. 1. Sequence arrangement in HSV DNA and distribution of viral genes in the HSV genome. The filled quadrangles represent terminal sequence **ab** and **ca** inverted and repeated internally to yield **b'a'c'**. See ref. 1 and Table 1 for details of genome structure and gene function. The latency associated transcripts (LATs) map within inverted repeats flanking U_L.

Genome Structure and Gene Content

The genome structure and current gene content of HSV-1 are summarized in Fig. 1. Exclusive of the variable number of repeats of the terminal **a** sequence, the HSV-1 genome is approximately 152 kbp in size (3, 4). The genome consists of two long stretches of quasi-unique sequences, unique long sequence (U_L) and unique short sequence (U_S), flanked by inverted repeats. U_L is flanked by the sequence **ab** and its inversion **b'a'**, approximately 9 kbp each, whereas U_S is flanked by the sequence **a'c'** and its inversion **ca**, 6.5 kbp each (5, 6). Thus, the HSV genome contains 15 kbp of DNA sequences (**b'a'c'**), which represent inverted repeats of terminal regions inserted between U_L and U_S domains. The **a** sequence varies in size and may be present in multiple copies adjacent to the **ba** sequence, but only in a single copy at the

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Abbreviations: HSV-1, -2, herpes simplex virus 1 and 2; ICP, infected cell protein; gB, gC, gD, etc., glycoproteins B, C, D, etc.; U_L, unique long sequence; U_S, unique short sequence; LAT, latency associated transcripts; ORF, open reading frame; CNS, central nervous system.

Table 1. The function of herpes simplex virus genes

Gene	Product	Dispensable in cell culture	Regulation	Function of gene product
$\gamma_1 34.5$	ICP34.5	Y	γ_1	Null mutants are attenuated and fail to block phosphorylation of eIF-2α by activated protein kinase RNA-dependent kinase; carboxyl terminus homologous to the corresponding domain of the GADD34 proteins.
ORF-P	ORF-P	Y	pre α	ORF is antisense to the $\gamma_1$34.5 gene and repressed by binding of ICP4 to cap site. Proteins interact with p32, a component of SF2/ASF splicing factor.
ORF-O	ORF-O	Y	pre α	Overlaps with ORF P, a protein made by frameshift from ORF-P.
α0	ICP0	Y	α	Promiscuous transactivator, requires ICP4 for optimal activity; nucleotidylylated, phosphorylated by U_L13, nuclear (early) and cytoplasmic (late) phases. Null mutants debilitated at low multiplicities of infection.
U_L1	gL	N	γ	Complex with gH required for transport of both proteins to plasma membrane and for viral entry mediated by gH.
U_L2		Y	β	Uracil DNA glycosylase.
U_L3		Y	γ_2	Nuclear phosphoprotein of unknown function. Reported to localize to perinuclear region early and to the nucleus late in infection.
U_L4		Y	Unknown	Unknown.
U_L5		N	β	Forms complex with U_L8 and U_L52 proteins.
U_L6		N	Unknown	Virion protein; required for DNA cleavage and packaging.
U_L7		N	Unknown	Unknown.
U_L8		N	β	Forms complex with U_L5 and U_L52 (helicase/primase complex). Stabilizes interaction between primers and DNA template.
U_L9		N	γ(?)	Binds to origins of DNA synthesis in sequence-specific (origin) fashion; carries out helicase and ATPase activities.
U_L10	gM	Y	γ	Glycoprotein present in virions and plasma membranes.
$U_L10.5$		?	Unknown	Unknown.
U_L11		Y	γ(?)	Myristoylated protein; necessary for efficient capsid envelopment and exocytosis.
U_L12		Y	β	Exonuclease (DNase) involved in viral nucleic acid metabolism; reported to localize in nucleoli and in virally induced nuclear dense bodies and to bind to *a* sequence along with other unidentified proteins. Complex may be involved in cleavage/packaging of viral DNA.
$U_L12.5$		Y	Unknown	Nuclease-associated with capsids.
U_L13		Y	γ	Virion (nuclear) protein kinase; substrates include ICP0, ICP22, *vhs*, U_L3, U_L49, etc.
U_L14		N	Unknown	Unknown.
U_L15		N	γ	*ts* mutant DNA+. Two exons; protein required for cleavage/packaging of DNA.
U_L16		Y	Unknown	Virion protein; gene located within intron of U_L15.
U_L17		N	γ	Located within intron of U_L15.
U_L18	VP23	N	γ	Protein required for capsid formation and cleavage/packaging of DNA.
U_L19	VP5, ICP5	N	γ_1	Major capsid protein.
U_L20		Y	γ	Membrane protein, associates with nuclear membranes, Golgi stacks, etc. Essential for viral exocytosis.
$U_L20.5$			γ_2	Unknown.
U_L21		Y	Unknown	Nucleotidylylated phosphoprotein; unknown function.
U_L22	gH	N	γ_2	Forms complex with gL (see above). Required for entry, egress, and cell–cell spread.
U_L23	ICP36	Y	β	Thymidine (nucleoside) kinase.
U_L24		Y	γ	Syn$^-$ locus; membrane-associated protein?
U_L25		N	γ	Virion protein reported to be required for packaging of cleaved viral DNA.
U_L26		N	γ	Serine protease; substrates are U_L26 protein and U_L26.5 (IC35). VP21 (C portion of U_L26), VP24 (N terminus of protease) are products of the self-cleavage of U_L26.
$U_L26.5$	ICP35	N	γ	Substrate of U_L26 protease unique to B capsids and forms inner core or scaffolding; the precursor, ICP35b,c is cleaved to e, f. On packaging of DNA it is removed from capsid shell.
U_L27	gB, VP7	N	γ_1	Glycoprotein required for viral entry; forms a dimer and induces neutralizing antibody. A *syn*$^-$ locus maps to the carboxyl terminus.
$U_L27.5$		?	Unknown	Unknown, antisense to gB.
U_L28	ICP18.5	N	γ	M_r 87–95 K protein required for DNA cleavage/packaging.
U_L29	ICP8	N	β	Binds single-stranded DNA cooperatively, required for viral DNA replication: forms complex with DNA polymerase and U_L42. *ts* mutants are DNA$^-$ and hence expression of early and late genes may be affected positively or negatively by ICP8. Because ICP8 denatures DNA, it affects renaturation of complementary strands of DNA and affects homologous pairing and strand transfer.
U_L30		N	β	DNA polymerase; forms complex with ICP8 and C terminal 247 amino acids of U_L42.
U_L31		N	γ_2	Nucleotidylylated phosphoprotein, cofractionates with nuclear matrix.
U_L32		N	γ_2	Cytoplasmic/nuclear protein required for DNA cleavage/packaging.
U_L33		N	Unknown	DNA packaging; necessary for assembly of capsids containing DNA.
U_L34		N	Unknown	Abundant nonglycosylated, membrane-associated, virion protein phosphorylated by U_S3.
U_L35	VP26	N	γ_2	Basic phosphorylated capsid protein.
U_L36	ICP1-2	N	γ_2	Tegument phosphoprotein. DNA is not released from capsids at nuclear pores in cells infected with *ts* mutant. Reported to form complex with a M_r 140 K protein that binds *a* sequence DNA.

Table 1. (Continued)

Gene	Product	Dispensable in cell culture	Regulation	Function of gene product
U_L37	ICP32	N	γ	Cytoplasmic phosphoprotein; in presence of ICP8 it is transported to nucleus and associates with DNA, but phosphorylation is not dependent on ICP8. Required for maturation of virions.
U_L38	VP19C	N	γ_2	Capsid assembly protein, binds DNA and may be involved in anchoring DNA in the capsid.
U_L39	ICP6	Y	β	Large subunit of ribonucleotide reductase. Autophosphorylates via unique N terminus but does not trans-phosphorylate.
U_L40		Y	β	Small subunit of ribonucleotide reductase.
U_L41	VHS	Y	γ	Causes nonspecific degradation of mRNA after infection; shuts off host protein synthesis, enables sequential synthesis of viral proteins.
U_L42		N	β	Double-stranded DNA-binding protein, binds to and increases processivity of DNA polymerase.
U_L43		Y	Unknown	Amino acid sequence predicts membrane-associated protein.
$U_L43.5$		Y		Antisense to U_L43; low abundance nuclear protein; accumulates in assemblons.
U_L44	gC, VP7.5	Y	γ_2	Glycoprotein involved in cell attachment; required for attachment to the apical surface of polarized MDCK cells.
U_L45		Y	γ_2	Encodes a M_r 18 K protein of unknown function.
U_L46	VP11/12	Y	γ	Tegument phosphoprotein reported to modulate the activity of U_L48 (aTIF).
U_L47	VP13/14	Y	γ_2	Nucleotidylylated tegument phosphoprotein modulates the activity of U_L48 (αTIF).
U_L48	VP16, ICP25, αTIF	N	γ	Tegument protein, induces α genes by interacting with *Oct*I. The complex binds to specific sequences with the consensus GyATGnTAATGArATTCyTTGnGGG-NC.
U_L49	VP22	N	γ	Nucleotidylylated, mono(ADP-ribosyl)ated tegument phosphoprotein.
$U_L49.5$		N	γ_2	Sequence predicts a M_r 12,000 membrane-associated protein.
U_L50		Y	β	dUTPase.
U_L51		Y	γ	Unknown.
U_L52		N	β	Component of the helicase/primase complex.
U_L53	gK	Y	γ	Glycoprotein required for efficient viral exocytosis; contains *syn*$^-$ locus.
$\alpha27$	ICP27	N	α	Nucleotidylylated multifunctional regulatory protein; causes redistribution of snRNPs, inhibits RNA splicing. It is required for late gene expression, and negatively regulates early genes.
U_L55		Y	Unknown	Unknown.
U_L56		Y	Unknown	Nuclear, virion-associated protein of unknown function.
$\alpha4$	ICP4	N	α	Nucleotidylylated, poly(ADP-ribosyl)ated phosphoprotein; regulates positively most β and γ genes and negatively itself, ORF-P and the $\alpha0$ gene; blocks apoptosis. Binds to DNA in sequence specific fashion.
$\alpha22$	ICP22	Y	α	Nucleotidylylated regulatory protein, phosphorylated by U_L13 and U_S3 protein kinases, required for optimal expression of ICP0 and of a subset of γ proteins.
$U_S1.5$	$U_S1.5$	Y	α	Regulatory protein; extent to which it shares function with ICP22 not known.
U_S2		Y	Unknown	Unknown.
U_S3		Y	β	Protein kinase; major substrate is U_L34 protein.
U_S4	gG	Y	γ	Glycoprotein involved in entry, egress, and spread from cell to cell.
U_S5	gJ(?)	Y	Unknown	Sequence predicts glycoprotein.
U_S6	gD VP17/18	N	γ_1	Glycoprotein required for post-attachment entry of virus into cells.
U_S7	gI	Y	γ	gI and gE glycoproteins form complex for transport to plasma membrane and also to constitute a high-affinity Fc receptor. gI is required for basolateral spread of virus in polarized cells.
U_S8	gE	Y	γ_2	FC receptor; involved in basolateral spread of virus in polarized cells.
$U_S8.5$		Y	β or γ_1	Unknown.
U_S9		Y	Unknown	Tegument protein phosphorylated by U_L13.
U_S10		Y	Unknown	Tegument protein.
U_S11		Y	γ_2	Tegument protein binds to U_L34 mRNA in sequence- and conformation-specific fashion; binds to the 60S ribosomal subunit and localizes in the nucleolus.
$\alpha47$	ICP47	Y	α	Binds to TAP1/TAP2 and to block antigen presentation to CD8$^+$ cells.
Ori_STU	Ori_SRNA	Y	γ_2	RNA transcribed across S origins of DNA synthesis. Function is not known.
LATU	LATs	Y	pre α?	Transcripts, found in latently infected nerons. Function is not known.

Ori_STU is the transcriptional unit across the origin of DNA synthesis in the S component. LATTU is the transcriptional unit expressed in latently infected sensory neurons. This table was updated and modified from Ref. 1. Additional references are as follows: $U_L10.5$, ref. 7; $U_L12.5$, ref. 15; $U_L20.5$, P. J. Ward and B.R. (unpublished data); $U_L27.5$, Y. Chang, G. Campadelli-Fiu , and B.R. (unpublished work); $U_L43.5$, ref. 14; $U_S1.5$, ref. 9; ORF-O, G. Randall and B.R. (unpublished work); U_S9, R. Brandimasti and B.R. (unpublished work).

terminus of the genome next to the c sequence and contains signals for cleavage of unit length DNA from concatemers and packaging of the DNA in preformed capsids (reviewed in ref. 1). HSV-1 is known to express at least 84 different polypeptides whose open reading frames (ORFs) are distributed as indicated in Fig. 1 (refs. 4 and 7–15; P. L. Ward and B.R., unpublished data; Y. Chang, G. Campadelli-Fiume, and B.R., unpublished data). Of this number, five ORFs, mapping in the inverted repeats, are present in two copies per viral genome. In addition to the ORFs listed in Fig. 1, infected cells contain

transcripts from genome domains not known to specify proteins. These include the LATs discussed below and an RNA (Ori_SRNA) derived by transcription of the two of the three origins of viral DNA synthesis mapping in inverted repeats (16, 17). The ORFs form several groups whose expression is coordinately regulated in a cascade fashion. The α genes are expressed first, functional α proteins are required for the expression of β genes, and both functional α proteins and viral DNA synthesis mediated by β proteins are required for (γ_2), or enhance (γ_1), the expression of late or γ genes (18, 19). Whereas α proteins perform regulatory functions or prevent a host response to infection, the function of β proteins is the management of the nucleic acid metabolism and viral DNA synthesis in the infected cell, as well as posttranslational modification of proteins made earlier and later in infection. The γ proteins are largely the structural components of the virions (reviewed in ref 1).

Since 1982 (20, 21) techniques have been available to delete or insert DNA sequences at specific sites. These studies have revealed the existence of ORFs that are expressed and the rather unexpected finding that 45 of the 83 ORFs specifying diverse proteins are dispensable for viral replication in at least some cells in culture. A list of the ORFs and the functions expressed by the gene products are shown in Table 1. The 38 ORFs that cannot be deleted without ablating the capacity of the virus to replicate include four genes specifying surface glycoproteins, two regulatory proteins [infected cell proteins no. 4 (ICP4) and no. 27 (ICP27)], seven proteins required for the synthesis of viral DNA, proteins required for assembly of the capsid, structural proteins, and proteins whose functions are not yet known. The 45 accessory ORFs, which are not required for viral replication in cells in culture, specify 11 proteins involved in entry, sorting, and exocytosis of virus (glycoproteins C, E, G, I, J, K, M; membrane proteins U_L11, U_L20, U_L24, U_L43), 2 protein kinases (U_L13, U_S3), 2 proteins that preclude host response to infection ($\alpha47$ and $\gamma_134.5$), 3 regulatory proteins ($\alpha0$, $\alpha22$, $U_S1.5$), 5 proteins that augment the nucleotide triphosphate pool or repair DNA (thymidine kinase, dUTPase, ribonucleotide reductase, DNase, uracil glycosylase), 1 protein that causes the degradation of mRNA after infection (U_L41), and numerous other proteins whose functions are not known (detailed references in ref. 1).

The Role of Selected Viral Genes in Viral Replication

The reproductive cycle of HSV has been described in detail elsewhere (1). The objective of viral replication is efficient, rapid synthesis and dissemination of viral progeny. In the process, the infected cell dies. Viral replication consists of a series of events very tightly regulated both positively and negatively. To accomplish its objectives, the virus brings into the newly infected cell several proteins packaged in the virion tegument (a layer of proteins located between the capsid and the envelope; see ref. 22), whose functions are best described as creating the environment for initiation of viral replication). One, designated as VP16 or α gene trans-inducing factor (αTIF) induces the transcription of α genes by cellular RNA pol II and accessory factors, whereas another encoded by U_L41 causes the degradation of cytoplasmic RNAs (23–25). The major regulatory protein ICP4 made after infection acts both negatively by binding to high-affinity sites on viral DNA and positively by an as yet unknown mechanism (reviewed in ref. 1). The hypothesis that ICP4 is directly involved in transcription is based on reports that it binds TATA box-binding protein and transcription factor IIB, and on the evidence that after the onset of DNA synthesis, it is a component of γ-transcriptons—nuclear stuctures containing newly synthesized viral DNA, RNA polymerase II, ICP22, and a cellular protein known as L22 or EAP and that is normally present in nucleoli and ribosomes and binds small RNA molecules (ref. 26; R. Leopardi, P. L. Ward, W. Ogle, and B.R., unpublished work). ICP27 has multiple functions, but primarily it regulates posttranscriptional processing of RNA (27). ICP22 also appears to be a transcriptional factor; it is required for the expression of the $\alpha0$ gene and also of a subset of γ genes and is a component of the γ-transcriptons (ref. 28; R. Léopardi, P. L. Ward, W. Ogle, and B.R., unpublished work). Among other proteins that regulate the replicative cycle is U_L13, a protein kinase known to mediate the phosphorylation of ICP0, ICP22, and other proteins (ref. 28; W. Ogle, K. Carter, and B.R., unpublished work). The function of another viral protein kinase, U_S3, is less clear (1).

The functions of $\gamma_134.5$ and $\alpha47$ genes are of particular interest. $\gamma_134.5$ appears to have at least two functions. One function of $\gamma_134.5$ is to preclude the shutoff of protein synthesis caused by activation of the protein kinase RNA-dependent kinase and, ultimately, by the phosphorylation of the α subunit of the translation initiation factor eIF-2 (29). The carboxyl-terminal domain of $\gamma_134.5$ required for this function is homologous to the corresponding domain of the mammalian protein GADD34—one of a set of proteins induced in growth arrest as a consequence of differentiation, serum deprivation, or DNA damage. Human GADD-34, or a chimeric gene consisting of the amino terminal domain of $\gamma_134.5$ and the carboxyl terminus of GADD-34, effectively replaces the $\gamma_134.5$ gene in the context of the viral genome (30). The second function of the $\gamma_134.5$ enables the virus to multiply efficiently in a number of tissues, but particularly in the CNS of experimental animal systems (31, 32). The argument that this function of $\gamma_134.5$ is independent of the function of the protein to preclude the phosphorylation of eIF-2α is based on the observation that viruses carrying GADD-34 in place of $\gamma_134.5$ are not blocked in protein synthesis; they are nevertheless attenuated (32).

$\alpha47$ binds the complex of TAP1/2 and thereby precludes the transport of peptides for presentation to $CD8^+$ cells (33).

The Function of Viral Genes in Latency

To date the only domain of the viral genome shown to be expressed during latency maps in the inverted repeats flanking U_L (16). The RNAs described to date consist of two populations. The low abundance population arises from an 8.3-kbp domain. The two abundant RNAs, of 2 and 1.5 kb respectively, and known by the acronym LAT, appear to be stable introns that accumulate in abundant amounts in nuclei of neurons harboring latent virus. Deletion of the upstream promoter or of the sequences encoding LATs has little effect on the establishment or maintenance of the latent state, but reduces the efficiency of latent virus to reactivate (1). LATs may be harbingers of neurons capable of reactivating than viral products required for establishment of latency. Given the multitude of viral accessory genes whose function is to render viral replication and dissemination more efficient, the notion that the virus depends solely on the cellular factors for dissemination seems unlikely. Recent studies have shown that the genome domain transcribed during latency contains ORF-0 and ORF-P, whose expression is repressed by ICP4, inasmuch as mutagenesis of the high-affinity binding site at the transcription initiation site of ORF-P led to the derepression of both genes (12). The virus carrying the derepressed gene is attenuated in experimental animal systems (mice) and underexpresses $\alpha0$ and $\alpha22$ proteins (34, 45). In addition, ORF-P protein colocalizes and binds to a protein (p34), which is a component of the SF2/ASF splicing factor (45). The role of ORF-P in latency is not known.

Genetic Engineering of Novel Viral Genomes

The two major techniques for construction of novel viruses depend on genetic recombination in infected or transfected cells. The first technique was based on the observation that transfection of cells with intact viral DNA and mutated fragment will result in a small fraction of the progeny carrying the mutated sequence (20, 21). To specifically select this population, the procedure first involved the insertion at or near the target for deletion by recombination through flanking sequences of the viral thymidine kinase as a selectable marker. Only the progeny of transfection, which carries the viral thymidine kinase gene, would multiply in thymidine kinase minus cells overlaid with appropriate medium. In the second step, the inserted thymidine kinase was deleted along with adjacent target sequences by recombination through flanking sequences with a mutated DNA sequence. In this instance, only thymidine kinase minus progeny would break through efficiently in cells overlaid with medium containing bromouracil deoxyriboside. A minor variant of this technique based on gene inactivation by random insertion of the mini-μu phage has the disadvantage in that the mini-μu DNA sequence is quite large and troublesome to remove (21).

The second procedure involves transfection of cells with overlapping cosmids containing appropriate insertions or deletions. Expression of genes contained in cosmids leads eventually through recombination to the reconstruction of full-length viral genomes. This procedure is less efficient, but the progeny of transfection need not be subjected to selection for the isolation of the desired genotype (35)

Both procedures suffer from gene rearrangements as a result of transfection. To link a specific genotype to the observed phenotype, it is essential to determine whether the wild-type phenotype is restored by the repair of the deleted sequences with a small DNA fragment. It is estimated that as much as 30% of the recombinants made by the techniques described above contain additional mutations detected only after the restoration of the missing sequence.

Requirements and Design of Viral Vectors

Vectors for delivery of cellular genes to CNS must not express viral genes that cause the infected cell to make cytotoxic viral proteins or that induce an immune response, which may damage the recipient cells. A huge literature describes attempts to obtain long-term expression of reporter genes in experimental animals, particularly mice. A potentially suitable vector for this type of application is based on construction of defective genomes, i.e., genomes that are unable to replicate in the recipient cells. In recent years, two different types of defective recombinant viruses have emerged. The first is based on defective HSV genomes, which arise spontaneously by recombination and are amplified during serial passage at high multiplicities (36). The defective genome subunit (the amplicon) consists minimally of the terminal **a** sequence and an origin of viral DNA synthesis. In virions, these unit are arranged head-to-tail. HSV-1 amplicons have long been shown to express efficiently cellular genes incorporated into them (37). Amplicons do not encode viral proteins and must therefore be supplied with both viral structural proteins and proteins required for viral DNA synthesis and exocytosis in order to be made. In theory, amplicons could accommodate as much as 150 kbp of DNA. In practice, three problems exist. First, until recently, amplicons were contaminated with helper DNA. Second, the amounts of amplicons made are not readily controllable and the usual yields are several orders of magnitude lower than those of wild-type infectious virus. Third, amplicons tend to be unstable on serial passage because the smaller the amplicon, the greater is its selective advantage (38). Of these problems, only the first one has been solved, since a helper virus incapable of packaging has been constructed (39). A significant potential problem is the rescue of the helper virus by recombination with the amplicon, which would enable the helper to package.

The second approach is based on construction of viruses lacking essential genes. The essential genes deleted singly or in combination include α4, α22, α27, U_L48 (α gene trans-inducing factor or VP16), and U_L41 (40). Deletion of the inverted repeats (**b'a'c'**) and of stretches of genes not essential for viral replication in cell culture (e.g., U_S except for U_S6, α22, and α47), and large stretches of U_L (U_L2, U_L3, U_L4, U_L10, U_L11, U_L43, U_L43.5, U_L45, U_L46, U_L47, U_L55, U_L56) could make space for insertion of at least 40 kbp of DNA. By necessity, the debilitated recombinant virus must be grown in cell lines expressing the deleted viral genes. This type of vector also presents several problems. Foremost is that some of the selected genes targeted for deletion perform a multiplicity of functions. For example, they may be both transactivators and repressors, and they normally block the cells from programmed cell death triggered by viral gene expression (41). It could be predicted therefore that sooner or later the recipient cell will cease functioning because of slow but ultimately fatal expression of viral genes. Other problems, perhaps more readily surmountable, include potential for recombination between the defective viral genomes and viral genes resident in the cell genome and the stability of the cell lines. In neither model is reactivation from latency a problem inasmuch as the viruses are unable to replicate.

Whereas gene therapy may require delivery of therapeutic genes to a significant fraction of cells that normally express them or could serve as surrogate expressors, cancer therapy requires complete destruction of cancer cells. Theoretically, for selective destruction of cancer cells, it should be sufficient to introduce a defective viral genome expressing a factor that is excreted in abundant amounts and toxic only for cancer cells. Theory not withstanding, viruses that infect or at least selectively multiply and destroy tumor cells are likely to have a therapeutic advantage. In experimental animal systems several genetically engineered viruses appear to have met at least the initial requirements for further development (32). The mutations that render the viruses attenuated fall into three categories. The first set encodes enzymes (e.g., thymidine kinase, ribonucleotide reductase, etc.) involved in nucleic acid metabolism and that would not be available in neurons but would be available to the virus in dividing tumor cells. The second category are genes encoding proteins (e.g., $\gamma_1$34.5), which disable the capacity of the virus to replicate in CNS for reasons not well understood, as described earlier in the text (reviewed in ref. 32). Lastly, deletion of inverted repeats (**b'a'c'**) in itself grossly debilitates HSV-1 (42, 43). Irrespective of the set of genes involved for attenuation, it will be virtually impossible to introduce virus in all cancer cells. As in the case of defective viruses, even replicating viruses will have to carry and express factors that induce immune response or activate pro-drugs selectively in cancer cells.

The use of recombinant viruses for prophylaxis against viral infection requires mutants that are genetically stable, incapable of replicating in CNS, incapable of spreading in immunocompromised individuals, unable to reactivate, not transmissible from immunized individual to contacts, but immunogenic and protective against disease caused by subsequent infection. A large number of defective viruses have been proposed for immunization on the grounds that although they cannot produce infectious progeny and fundamentally make proteins only in the set of initially infected cells, the presentation to the immune system of viral antigens made in these cells is superior to that of subunit vaccines (e.g. ref. 44). The central issue is the immunogenic mass required for effective immunity and whether such a mass could be achieved by progeny of a replication-defective virus. Secondary issues are the stability of

the cell lines expressing the complementing viral genes, rescue of the defective virus by recombination, and qualification of transformed cell lines for administration of virus along with cellular DNA to healthy individuals. The construction of replicating virus is not less daunting, and parallels in many respects the construction of recombinant viruses for cancer therapy.

Conclusions

Development of genetic engineering evolved *pari passu* with our knowledge of viral gene content and function and is at a point where, subject to constraints imposed by the size of the virion, the construction of virtually any vector is feasible. The outstanding issues are not the construction of viral vectors, but rather (*i*) delivery of recombinant viruses to appropriate cells, (*ii*) regulation of expression of the gene carried by the viral vector, and (*iii*) regulatory issues related to qualification of continuous cell lines, which express complementing viral genes for replication of defective vectors. Theoretically, it should be possible to create viruses that carry surface protein destined for receptors present only on a specific set of cells, but this is not yet feasible. The problems associated with regulation of gene expression are particularly vexing because the available size for packaging genes in the viral genome is too small to accommodate the genomic versions rather than the cDNA version of most cellular genes of interest. As a consequence, alternative splicing to produce isoforms of cellular proteins and natural regulation of gene expression may not be feasible. The third issue arises from the difficulty of purifying virus away from cell debris which contains cellular DNA. Since only continuous cell lines are likely to express stably viral genes complementing replication defective viruses, it would be necessary to define the requirements for the qualification of such cell lines for production of viruses intended for human use. Considering the progress of the past 10 years, the solution of these problems should not be far behind.

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Exhibit B: Bowers et al., "Expression of vhs and VP16 During HSV-1 Helper Virus-Free Amplicon Packaging Enhances Titers," *Gene Therapy* 8(2):111-120 (2001)

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RESEARCH ARTICLE

Expression of vhs and VP16 during HSV-1 helper virus-free amplicon packaging enhances titers

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Recently developed helper virus-free methods of herpes simplex virus (HSV) amplicon vector packaging provide stocks that are virtually devoid of the cytotoxic component normally associated with traditional helper virus-based packaging methods. These approaches involve cotransfection of amplicon plasmid DNA with either a five-cosmid set or a bacterial artificial chromosome (BAC) that contains the HSV genome without its cognate pac signals. Helper virus-free amplicon packaging produces low-titer stocks (<10^5 expressing particles/ml) that exhibit a high frequency of pseudotransduction. In an effort to enhance amplicon titers, we introduced in trans a genomic copy of the virion host shutoff (vhs) protein-encoding gene UL41 into both cosmid- and BAC-based packaging strategies. Cotransfection of this plasmid with the amplicon and packaging reagents results in a 10-fold higher amplicon titer, and stocks that do not exhibit the pseudotransduction phenomenon. To further enhance packaging efficiency, the HSV transcriptional activator VP16 was introduced into packaging cells 1 day before the packaging components. Pre-loading of packaging cells with VP16 led to an additional enhancement of amplicon titers, an effect that did not occur in the absence of vhs. Increased helper virus-free amplicon titers resulting from these modifications will make in vivo transduction experiments more feasible.
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Keywords: *HSV; amplicon; gene therapy; titer; GFP; β-galactosidase*

Introduction

The herpes simplex virus (HSV)-derived amplicon vector is an attractive gene delivery tool for the central nervous system due to its ease of manipulation, large transgene capacity, and natural neurotropism.[1–5] Efforts to bring this vector system into the clinical arena for treating neurodegenerative diseases have been hampered by potential cytotoxicities that are associated with traditional methods of virus packaging. This problem mainly involves the copackaging of helper virus that encodes cytotoxic and immunogenic viral proteins. Newer methods of packaging have been developed that result in 'helper virus-free' amplicon stocks.[6,7] Stocks produced by these means, however, are typically of low titer (<10^5 expression units/ml), allowing for modest scale *in vitro* experimentation. Such low titers discourage investigators from performing the large animal studies required to develop and assess potential amplicon-directed therapies in humans.

Not only do early generation helper virus-free packaging strategies lead to lower amplicon titers than traditional methods, they also produce stocks that exhibit a high frequency of pseudotransduction events when used to infect a variety of cell types. Viral stock-derived gene product appears as punctate material localized to cellular vesicles, instead of throughout the cell as is observed following vector-directed *de novo* expression. This phenomenon is either a consequence of vector transgene product from packaging cells that co-purifies with the virus or is the product of inefficient amplicon packaging. Pseudotransduction is not readily observed with stocks derived from helper virus-based strategies, suggesting that optimal packaging conditions do not yet exist for helper virus-free strategies.

Optimal propagation of wild-type HSV virions requires orderly progression of α, β, and γ gene transcription following infection of a host cell. This is achieved by delivery of copackaged proteins carried by the virion that facilitate co-opting of cellular transcription machinery and transactivation of viral α gene promoters. Therein lie the fundamental differences that exist between helper virus-free packaging and traditional helper virus-based packaging methods. Helper virus-based packaging involves superinfection of an amplicon DNA-transfected monolayer of packaging cells with a replication-defective helper virus. The helper virus genome, as in the case of wild-type HSV, is delivered to the cell in a complex with copackaged proteins, including VP16 and virion host shutoff (vhs). The HSV vhs protein functions to inhibit the expression of genes in infected cells via destabilization of both viral and host mRNAs. Because vhs plays such a vital role in establishment of the HSV replicative cycle and as a potential structural protein, its presence early during amplicon packaging may account for the higher titers observed for helper virus-based packaging. VP16 is another important

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copackaged protein that resides in the helper virus nucleocapsid and is responsible for activating transcription of HSV immediate–early genes to initiate the cascade of lytic cycle-related viral protein expression.

Helper virus-free methods of amplicon packaging, conversely, involve cotransfection of naked DNA forms of either a HSV genome-encoding cosmid set or BAC reagent with an amplicon vector. In this instance, the HSV genome gains access to the cell without copackaged vhs or VP16. The initiation and temporal progression of HSV gene expression is potentially not optimal for production of packaged amplicon vectors due to the absence of these important HSV proteins.

Because helper virus-free packaging reagents are delivered to a host cell without important viral regulatory proteins, the efficiency of amplicon packaging may likely be increased by introduction of vhs and VP16 during the initial phase of virus propagation. To this end, a vhs-encoding DNA segment was included in the packaging protocol as a cotransfection reagent. Additionally, packaging cells were 'pre-loaded' with VP16 to mimic its presence during helper virus-mediated amplicon packaging. These modifications in aggregate led to a 30- to 50-fold enhancement of packaged amplicon vector titers, nearly approximating titers obtained using helper virus-based traditional approaches. The resultant viral stocks also did not exhibit the pseudotransduction phenomenon. Improvements such as these to helper virus-free techniques of HSV amplicon vector production increase the likelihood that large-scale *in vivo* applications of amplicon vectors will be attainable.

Results

The full potential of HSV amplicon vectors for therapeutic gene delivery has not been realized. Widespread use of this vector system has been hampered by helper virus-mediated cytotoxicity associated with traditionally packaged amplicon stocks or by the low titers obtained from helper virus-free production methods. Helper virus-free methods of packaging hold the most promise as resultant stocks exhibit little or no cytotoxicity. Modifications to such packaging strategies could be made to increase viral titers.

Our laboratory has utilized both cosmid- and BAC-based methods of helper virus-free packaging previously described.[6–8] The low titers observed for helper virus-free methods may be a result of the sub-optimal state of the HSV genome at the beginning of amplicon production, as the genome is without copackaged viral regulators vhs and VP16. To determine if introduction of vhs into the packaging scheme could increase amplicon titers and quality, we cloned a genomic segment of the UL41 gene into pBluescript and added this plasmid (pBSKS(vhs)) to the cotransfection protocols to provide vhs *in trans*. The genomic copy of UL41 contained the transcriptional regulatory region and flanking *cis* elements believed to confer native UL41 gene expression during packaging. When pBSKS(vhs) was added to the packaging protocols for production of a β-galactosidase (*lacZ*)-expressing amplicon (HSVlac), a maximum of 10-fold enhanced amplicon expression titers was observed for both cosmid- and BAC-based strategies (Figure 1a and b, respectively). As observed previously, the expression titers for HSVlac virus produced by the BAC-based method were approximately 500- to 1000-fold higher than stocks produced using the modified cosmid set. Even though titers were disparate between the differently prepared stocks, the effect of additionally expressed vhs on amplicon titers was analogous.

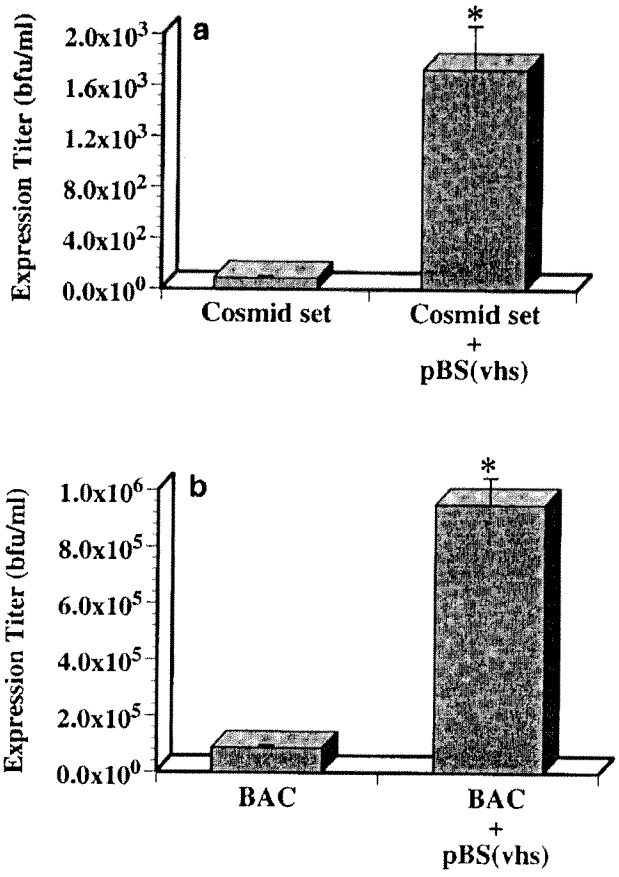
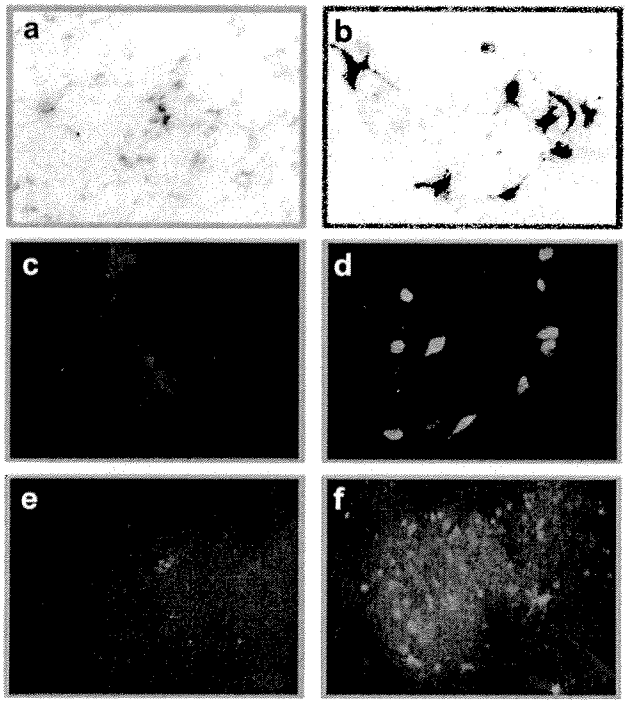


Figure 1 Addition of vhs enhances helper virus-free amplicon packaging titers. The β-galactosidase-expressing (LacZ) HSV amplicon vector (HSVlac) was packaged in the absence or presence of pBS(vhs) by either the cosmid- (a) or BAC-based (b) helper virus-free production strategy. This pBS(vhs) plasmid possesses the vhs open reading frame as well as its entire 5′ and 3′ regulatory sequences. Amplicon stocks were harvested and used to transduce NIH 3T3 cells, and titers were determined 1 day later via enumeration of LacZ-positive cells. Titer data are expressed as blue-forming units per milliliter (bfu/ml) and error bars represent standard deviation. The asterisk indicates a statistically significant difference ($P < 0.001$) between packaging reagent alone and packaging reagent plus the pBS(vhs) plasmid.

The punctate appearance of reporter gene product (pseudotransduction), a phenomenon associated with first-generation helper virus-free stocks, was substantially diminished *in vitro* when vhs was included in BAC-based packaging of a β-galactosidase-expressing (HSVlac; Figure 2a and b) or an enhanced green fluorescent (GFP)-expressing virus (HSVPrPUC/CMVegfp; Figure 2c and d). Pseudotransduction was not observed, as well, for cosmid-packaged amplicon stocks prepared in the presence of vhs (data not shown). To assess the ability of the improved amplicon stocks to mediate gene delivery *in vivo*, BAC-packaged HSVPrPUC/CMVegfp virus prepared in the absence or presence of pBSKS(vhs) was injected stereotactically into the striata of C57BL/6



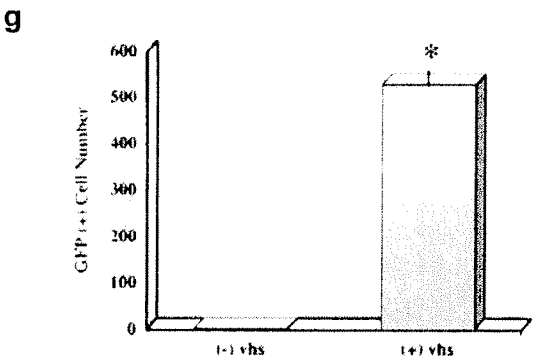


Figure 2 In vitro and in vivo analysis of vhs-mediated enhancement of helper-free amplicon titers. To demonstrate the effect of ectopic vhs expression on pseudotransduction, 10 ml of BAC-packaged HSVlac produced either without (a) or in the presence of pBS(vhs) (b) was used to transduce NIH 3T3 fibroblasts. LacZ-positive cells were visualized by X-gal histochemistry and images were digitally acquired. Ten microliters of BAC-packaged HSVPrPUC/CMVegfp produced either without (c) or in the presence of pBS(vhs) (d) was also used to transduce NIH 3T3 fibroblasts. Green fluoresecent protein (GFP)-positive cells were visualized with a fluorescent microscope and images digitally acquired. Three microliters of the same HSVPrPUC/CMVegfp virus samples packaged either in the absence (e) or in the presence of pBS(vhs) (f) was stereotactically delivered into the striata of C57BL/6 mice. Animals were killed 4 days later and prepared for visualization and quantitation of GFP-positive cells. Images used for morphological analyses were digitally acquired at 200 × magnification on 40-μm sections. All compartments were processed for cell counting and GFP-positive cell numbers reflect cell counts throughout the entire injection site (g). The asterisk indicates a statistically significant difference ($P < 0.001$) between amplicon stocks packaged with BAC alone and those packaged with BAC in the presence of pBS(vhs).

mice. Four days following infection, animals were killed and analyzed for GFP-positive cells present in the striatum (Figure 2e and f). The numbers of cells transduced by HSVPrPUC/CMVegfp prepared in the presence of vhs were significantly higher than in animals injected with stocks produced in the absence of vhs (Figure 2g). In fact, it was difficult to identify GFP-positive cells definitively in animals transduced with vhs(–) amplicon stocks.

The mechanism by which vhs expression resulted in higher apparent amplicon titers in helper virus-free packaging could be attributed to one or several properties of vhs. The UL41 gene product is a component of the viral tegument and could be implicated in structural integrity, and its absence could account for the appearance of punctate gene product material following transduction. For example, the viral particles may be unstable as a consequence of lacking vhs. Thus, physical conditions, such as repeated freeze–thaw cycles or long-term storage, may have led to inactivation or destruction of vhs-lacking virions at a faster rate than those containing vhs.

The stability of HSVPrPUC/CMVegfp packaged via the BAC method in the presence or absence of vhs was analyzed initially with a series of incubations at typically used experimental temperatures. Viral aliquots from prepared stocks of HSVPrPUC/CMVegfp were incubated at 4, 22 or 37°C for periods up to 3 h. Virus recovered at time points 0, 30, 60, 120 and 180 min were analyzed for their respective expression titer on NIH 3T3 cells. The rates of decline in viable amplicon particles, as judged by their ability to infect and express GFP, did not differ significantly between the vhs(+) and vhs(–) stocks (Figure 3a–c). Another condition that packaged amplicons encounter during experimental manipulation is freeze–thaw cycling. Repetitive freezing and thawing of virus stocks is known to diminish numbers of viable particles, and potentially the absence of vhs in the tegument of BAC-packaged amplicons leads to sensitivity to freeze fracture. To test this possibility, viral aliquots were exposed to a series of four freeze–thaw cycles. Following each cycle, samples were removed and titered for GFP expression on NIH 3T3 cells as described previously. At the conclusion of the fourth freeze–thaw cycle, the vhs(–) HSVPrPUC/CMVegfp stock exhibited a 10-fold diminution in expression titers as opposed to only a two-fold decrease for vhs(+) stocks (Figure 3d). This observation suggests that not only do vhs(+) stocks have increased expression titers, but the virions are more stable when exposed to temperature extremes, as determined by repetitive freeze–thaw cycling.

The native HSV genome enters the host cell with several viral proteins besides vhs, including the strong transcriptional activator VP16. Once within the cell, VP16 interacts with cellular transcription factors and HSV genome to initiate immediate–early gene transcription. Under helper virus-free conditions, transcriptional initiation of immediate–early gene expression from the HSV genome may not occur optimally, thus leading to lower than expected titers. To address this issue, a VP16 expression construct was introduced into packaging cells before cosmid/BAC, amplicon, and pBSKS(vhs) DNAs, and resultant amplicon titers were measured. To achieve regulated expression a glucocorticoid-controlled VP16 expression vector was used ($pGRE_5vp16$).

The $pGRE_5vp16$ vector was introduced into the pack-

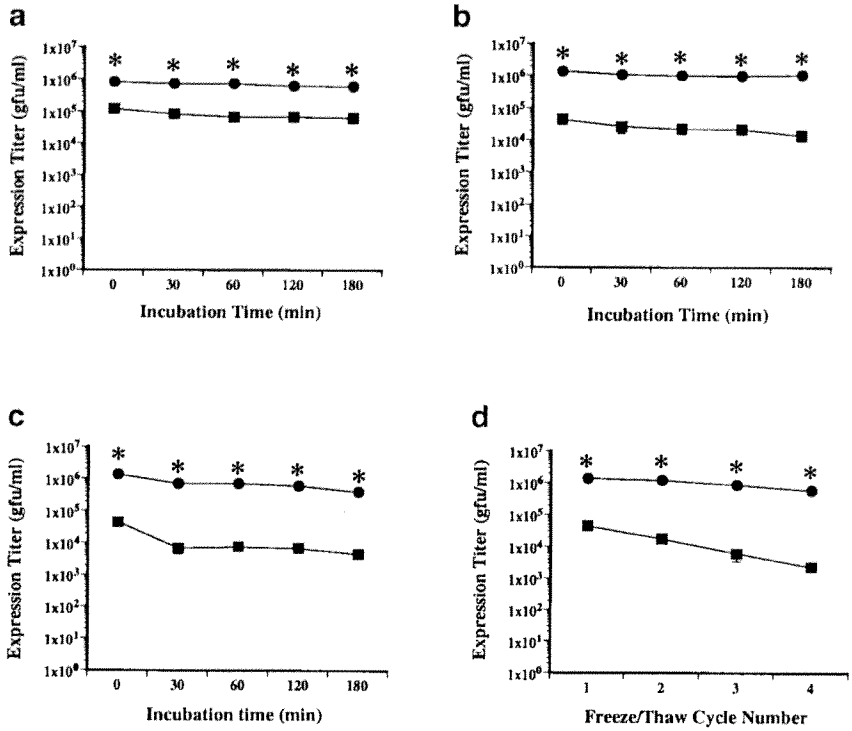


Figure 3 Presence of vhs during amplicon packaging imparts freeze/fracture stability, but does not enhance thermostability. BAC-packaged HSVPrPUC/CMVegfp stocks produced in the presence (circles) or absence (squares) of vhs were incubated at 0°C (a), 22°C (b), or 37°C (c) for varying time periods. At 0, 30, 60, 120 and 180 min following initiation of the incubations, aliquots were removed, titered on NIH 3T3 cells, and expression titer data represented as green-forming units per milliliter. Another set of HSVPrPUC/CMVegfp stocks were subjected to a series of freeze–thaw cycles to determine sensitivity of viral particles to freeze fracture. Following each cycle, aliquots were removed, titered on NIH 3T3 cells, and expression titer data represented as green-forming units per milliliter (gfu; d). The asterisk indicates a statistically significant difference (RMANOVA, $P < 0.001$) between amplicon stocks packaged with BAC alone and those packaged with BAC in the presence of pBS(vhs).

aging cells 24 h before transfection of the regular packaging DNAs. HSVlac was packaged in the presence or absence of vhs and/or VP16 and resultant amplicon stocks were assessed for expression titer. Some packaging cultures received 100 nM dexamethasone at the time of $pGRE_5vp16$ transfection to induce VP16 expression strongly; others received no dexamethasone. Introduction of $pGRE_5vp16$ in an uninduced (basal levels) or induced state (100 nM dexamethasone) had no effect on HSVlac titers when vhs was absent from the cosmid- or BAC-based protocol (Figure 4a and b). In the presence of vhs, addition of $pGRE_5vp16$ led to either a two- or five-fold enhancement of expression titers over those of stocks packaged with only vhs (cosmid- and BAC-derived stocks, respectively; Figure 4a and b). The effect of 'uninduced' $pGRE_5vp16$ on expression titers suggested that VP16 expression was occurring in the absence of dexamethasone. To examine this, Western blot analysis with a VP16-specific monoclonal antibody was performed using lysates prepared from BHK cells transfected with the various packaging components. Cultures transfected with $pGRE_5vp16$/BAC/pBSKS(vhs) in the absence of dexamethasone did show VP16 levels intermediate to cultures transfected either with BAC alone (lowest) or those transfected with $pGRE_5vp16$/BAC/pBSKS(vhs) in the presence of 100 nM dexamethasone (highest) (Figure 4c). There was no difference in level of $pGRE_5vp16$-mediated expression in the presence or absence of BAC, nor did dexamethasone treatment induce VP16 expression from the BAC (data not shown).

VP16-mediated enhancement of packaged amplicon expression titers could be due to increased DNA replication and packaging of amplicon genomes. Conversely, the additional VP16 that is expressed via $pGRE_5vp16$ could be incorporated into virions and act by increasing vector-directed expression in transduced cells. To test the possibility that VP16 is acting by increasing replication in the packaging cells, concentrations of vector genomes in BAC-derived vector stocks were determined. HSVlac stocks produced in the presence or absence of vhs and/or VP16 were analyzed using a 'real-time' quantitative PCR method. The concentration of vector genome was increased two-fold in stocks prepared in the presence of VP16 and this increase was unaffected by the presence of vhs (Figure 5).

There is a possibility that addition of viral proteins, like vhs and VP16, to the packaging process may lead to vector stocks that are inherently more cytotoxic. The amplicon stocks described above were examined for cytotoxicity using a lactate dehydrogenase (LDH) release-based cell viability assay. Packaged amplicon stocks were used to transduce NIH 3T3 cells and 48 h following infection, viability of the cell monolayers was assessed by the LDH-release assay. Amplicon stocks produced in the

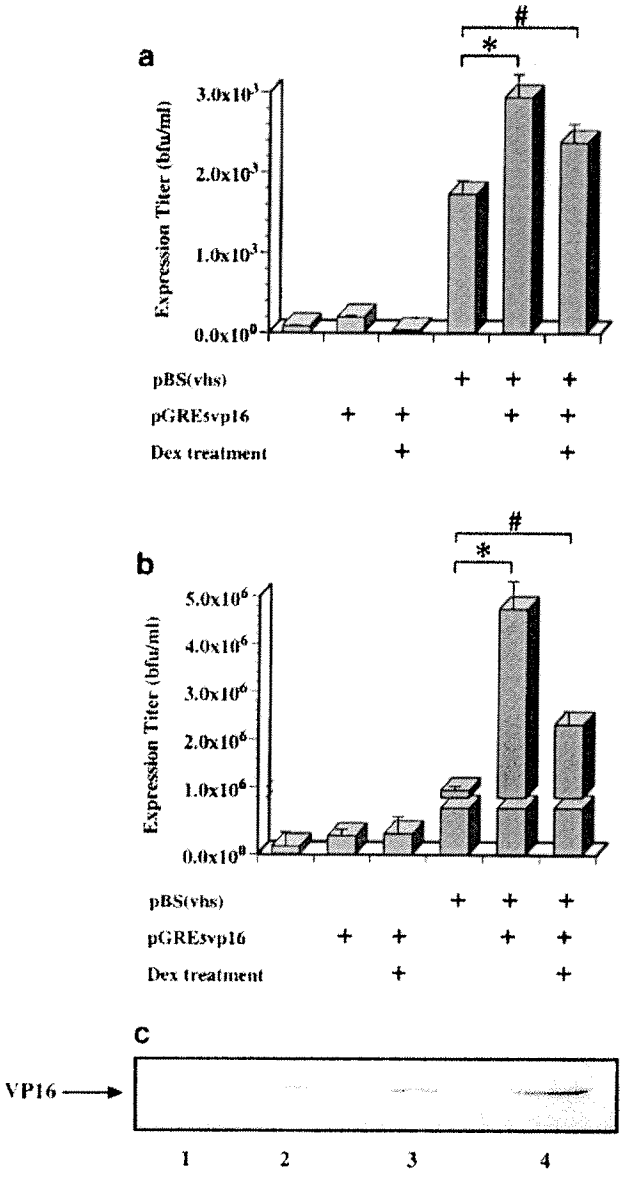


Figure 4 Preloading of packaging cells with VP16 leads to a two- to five-fold additional enhancement of amplicon expression titers only in the presence of vhs. BHK cells were plated and 6 h later, were transfected with a glucocorticoid-regulated VP16 expression vector (pGRE$_5$vp16). A subset of cultures received 100 nM dexamethasone following the VP16 plasmid transfection. The following day, HSVlac, a β-galactosidase-expressing amplicon, was cosmid- (a) or BAC-packaged (b) in the absence or presence of the pBS(vhs) plasmid using the modified BHK cultures. Resultant amplicon stocks were titered on NIH 3T3 cells using X-gal histochemistry and titers represented as blue-forming units per milliliter (bfu/ml; a and b). Error bars represent standard deviation. The asterisk indicates a statistically significant difference (a, $P < 0.05$; b, $P < 0.001$) between amplicon stocks prepared with packaging reagent plus pBS(vhs) and those made in the presence of packaging reagent, pBS(vhs), and pGRE$_5$vp16. The hash sign indicates a statistically significant difference (a, $P < 0.05$; b, $P < 0.001$) between amplicon stocks prepared with packaging reagent plus pBS(vhs) and those made in the presence of packaging reagent, pBS(vhs), pGRE$_5$vp16, and 100 nM dexamethasone. Western blot analysis was performed to determine levels of VP16 expression in various combinations of helper virus-free packaging components (c). Lysates were harvested 48 h following introduction of BAC reagent. Lane designations are the following: BHK cells alone (lane 1); BHK cells transfected with BAC only (lane 2); BHKs transfected with pGRE$_5$vp16 24 h before BAC transfection in the absence of dexamethasone (lane 3); and BHKs transfected with pGRE$_5$vp16 24 h before BAC transfection in the presence of 100 nM dexamethasone (lane 4). The 65-kDa VP16 protein was detected using a VP16-specific monoclonal antibody and goat anti-mouse secondary antibody in combination with a chemiluminescent detection kit.

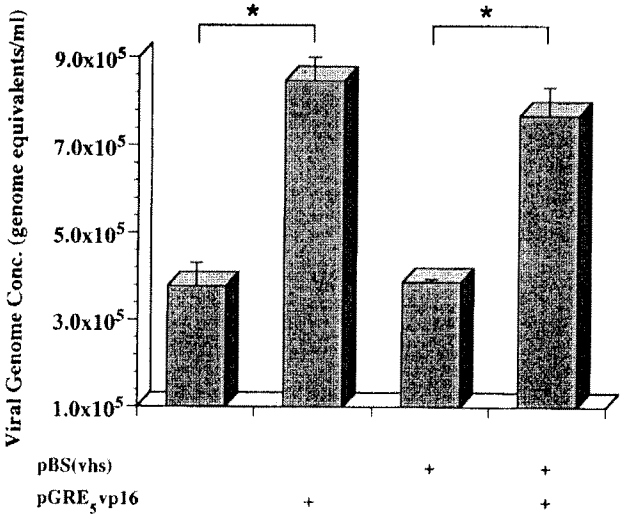


Figure 5 Virion-incorporated amplicon genome levels are enhanced by ectopic expression of VP16. BAC-packaged HSVlac stocks prepared in the presence or absence of VP16 and/or vhs were analyzed for levels of genome content using a 'real-time' quantitative PCR technique. Nanogram quantities of vector genome were assayed for each sample and data were expressed as detected amplicon genome per milliliter. Error bars represent standard deviation. The asterisk indicates a statistically significant difference ($P < 0.001$) between amplicon stocks packaged without pGRE$_5$vp16 and those packaged in the presence of this VP16 expression construct.

presence of vhs and VP16 displayed less cytotoxicity on a per virion basis than stocks packaged using the previously published BAC-based protocol (Figure 6).[7]

Discussion

Wild-type HSV virions contain multiple regulatory proteins that prepare an infected host cell for virus propagation. These virally encoded regulators, which are localized to the tegument and nucleocapsid, include vhs and VP16, respectively. The UL41 gene-encoded vhs protein exhibits an essential endoribonucleolytic cleavage activity during lytic growth that destabilizes both cellular and viral mRNA species.[9] Vhs-mediated ribonucleolytic activity appears to prefer the 5′ ends of mRNAs over 3′ termini, and the activity is specific for mRNA, as vhs does not act upon ribosomal RNAs.[10] Vhs also serves a structural role in virus particle maturation as a component of the tegument. HSV isolates that possess disruptions in UL41 demonstrate abnormal regulation of IE gene transcription and significantly lower titers than wild-type HSV-1,[11] presumably due to the absence of vhs activity. Therefore, because vhs is essential for efficient production of viable wild-type HSV particles, it likely plays a similarly important role in packaging of HSV-1-derived amplicon vectors.

The term 'pseudotransduction' refers to virion expression-independent transfer of biologically active

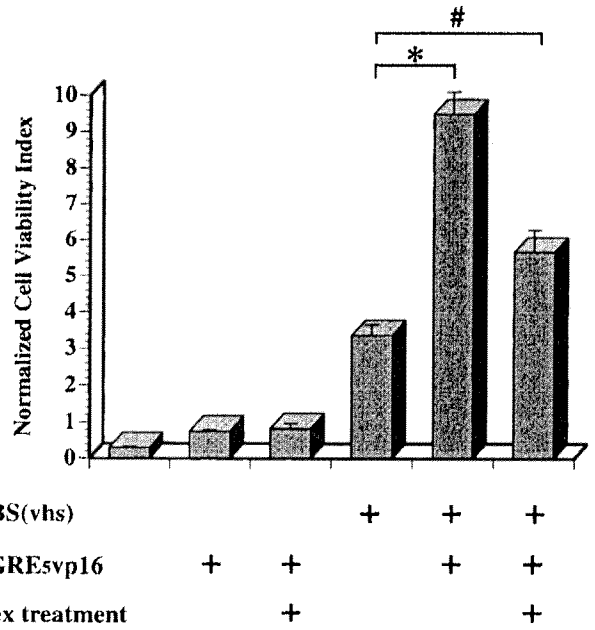


Figure 6 Amplicon stock-mediated cytotoxicity is not increased by additional expression of vhs and VP16 during packaging. BAC-packaged HSVlac stocks prepared in the presence or absence of VP16 and/or vhs were analyzed on confluent monolayers of NIH 3T3 cells for elicited cytotoxicity as determined by an LDH release-based assay. Two of the packaging samples that received pGRE$_5$vp16 were also treated with 100 nM dexamethasone 24 h before the packaging transfection. Equivalent expression units of virus from each packaging sample were used in the transductions. Viability data were represented as normalized cell viability index. Error bars represent standard deviation. The asterisk indicates a statistically significant difference ($P < 0.001$) between amplicon stocks prepared with BAC plus pBS(vhs) and those made in the presence of BAC, pBS(vhs), and pGRE$_5$vp16. The hash sign indicates a statistically significant difference ($P < 0.001$) between amplicon stocks prepared with BAC plus pBS(vhs) and those made in the presence of BAC, pBS(vhs), pGRE$_5$vp16, and 100 nM dexamethasone.

vector-encoded gene product to target cells.[12–14] This phenomenon was originally described with retrovirus and adeno-associated virus vector stocks and was shown to result in an overestimation of gene transfer efficiencies. β-Galactosidase and alkaline phosphatase are two commonly expressed reporter proteins that have been implicated in pseudotransduction, presumably due to their relatively high enzymatic stability and sensitivity of their respective detection assays.[13] Stocks of β-galactosidase expressing HSVlac and GFP-expressing HSVPrPUC/CMVegfp exhibited high levels of pseudotransduction when packaged in the absence of vhs (Figure 2 and unpublished observations). Upon addition of vhs to the previously described helper virus-free packaging protocols,[6,7] a 10-fold increase in expression titers and concomitant decrease in pseudotransduction were observed *in vitro*.

Vhs-mediated enhancement of HSV amplicon packaging was even more evident when stocks were examined *in vivo*. GFP-expressing cells in animals transduced with vhs(+) stocks were several hundred-fold greater in number than in animals receiving vhs(−) stocks. This could have been due to differences in virion stability, where decreased particle stability could have led to release of copackaged reporter gene product observed in the case of vhs(−) stocks. Additionally, the absence of vhs may have resulted in packaging of reporter gene product into particles that consist of only tegument and envelope.[15] Release of copackaged reporter gene product in either case could potentially activate a vigorous immune response in the CNS, resulting in much lower than expected numbers of vector-expressing cells.

Pre-loading of packaging cells with low levels of the potent HSV transcriptional activator VP16 led to a two- to five-fold additional increase in amplicon expression titers only in the presence of vhs for cosmid- and BAC-based packaging systems, respectively. This observation indicates the transactivation and structural functions of VP16 were not sufficient to increase viable viral particle production when vhs was absent, and most likely led to generation of incomplete virions containing amplicon genomes as detected by quantitative PCR. When vhs was present for viral assembly, however, VP16-mediated enhancement of genome replication led to higher numbers of viable particles formed. Quantitative PCR analysis of amplicon stocks produced in the presence of VP16 and vhs showed that viral genomes were increased only two-fold while expression titers were increased five-fold over stocks produced in the presence of vhs only (Figure 5). This result suggests that a portion of the effect related to VP16-mediated enhancement of genome replication while the additional two-fold enhancement in expression titers may be attributed to the structural role of VP16. The effect of VP16 on expression titers was not specific to amplicons possessing the immediate–early 4/5 promoter of HSV, as amplicons with other promoters were packaged to similar titers in the presence of VP16 and vhs (Bowers *et al*, unpublished observations).

VP16 is a strong transactivator protein and structural component of the HSV virion.[16] VP16-mediated transcriptional activation occurs via interaction of VP16 and two cellular factors, Oct-1[17–19] and HCF,[20,21] and subsequent binding of the complex to TAATGARAT elements found within HSV IE promoter regions.[22] This interaction results in robust up-regulation of IE gene expression. Neuronal splice-variants of the related Oct-2 transcription factor have been shown to block IE gene activation via binding to TAATGARAT elements,[23] suggesting that cellular transcription factors may also play a role in limiting HSV lytic growth.

The levels of VP16 appear to be important in determining its effect on expression titers. Low, basal levels of VP16 (via uninduced pGRE$_5$vp16) present in the packaging cell before introduction of the packaging components induced the largest effect on amplicon expression titers. Conversely, higher expression of VP16 (via dexamethasone-induced pGRE$_5$vp16) did not enhance virus production to the same degree and may have, in fact, abrogated the process. The presence of glucocorticoids in the serum components of growth medium is the most likely reason for this low-level VP16 expression, as charcoal-stripped sera significantly reduces basal expression from this construct (Bowers *et al*, unpublished observation). Perhaps only a low level or short burst of VP16 is required to initiate IE gene transcription, but excessive VP16 leads to disruption of the temporal progression through the HSV lytic cycle, possibly via inhibition of vhs activity. Moreover, evidence has arisen to suggest vhs activity is down-regulated by interaction with newly synthesized VP16 during the HSV lytic cycle,

thereby allowing for accumulation of viral mRNAs after host transcripts have been degraded.[24–26] Therefore, a delicate regulatory protein balance may be required to attain optimal infectious particle propagation. Additionally, the 100 nM dexamethasone treatment used to induce VP16 expression may have a deleterious effect on cellular gene activity and/or interfere with replication of the OriS-containing amplicon genome in packaging cells. High levels of dexamethasone have been shown previously to repress HSV-1 OriS-dependent replication by an unknown mechanism.[27] Inhibition of OriS-dependent replication does not appear to be responsible for our results, however, since quantitative PCR analysis of amplicon stocks produced in the presence and absence of dexamethasone indicated no change in genome content as a function of drug concentration (Figure 5 and data not shown). It is interesting to note that amplicon stocks were prepared in the presence of hexamethylene bisacetamide (HMBA). HMBA has been shown to compensate for the absence of VP16, thus leading to the transactivation of immediate–early gene promoters.[28] In the absence of HMBA pre-loading a packaging cell with VP16 could impart an even more dramatic effect on titers.

Ectopic expression of vhs and VP16 did not lead to amplicon stocks that exhibited higher cytotoxicity than helper virus-free stocks prepared in the traditional manner when examined by an LDH-release assay. Stocks prepared by the various methods were equilibrated to identical expression titers before exposure to cells. The heightened cytotoxicity in stocks produced in the absence of vhs and/or VP16 may reflect that larger volumes of these stocks were required to obtain similar expression titers as the vhs/VP16-containing samples or the levels of defective particles in the former may be significantly higher. Contaminating cellular proteins that copurify with the amplicon particles are most likely higher in concentration in the traditional stocks, and probably impart the higher toxicity profiles observed.

We recognize that further improvements in HSV amplicon production, purification, and assurances of vector safety are required before this gene delivery system can be utilized in the clinic. However, the modifications described here represent a major step forward toward realization of this goal.

Materials and methods

Cell culture

Baby hamster kidney (BHK) cells were maintained as described before.[29] The NIH-3T3 mouse fibroblast cell line was originally obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin.

Plasmid construction

The HSVPrPUC/CMVegfp amplicon plasmid was constructed by cloning the 0.8-kb cytomegalovirus (CMV) immediate–early promoter and 0.7-kb enhanced green fluorescent protein cDNA (Clontech, Palo Alto, CA, USA) into the *Bam*HI restriction enzyme site of the pHSVPrPUC amplicon vector.[30] A 3.5 kb *Hpa*I/*Hin*dIII fragment encompassing the UL41 (*vhs*) open reading frame and its 5′ and 3′ transcriptional regulatory elements was removed from *cos56*[31] and cloned into pBSKSII (Stratagene, La Jolla, CA, USA) to create pBSKS(vhs). For construction of $pGRE_5vp16$ the VP16 coding sequence was amplified by PCR from pBAC-V2 using gene-specific oligonucleotides that possess *Eco*RI (5′-CGGAATTCCGCAGGTTTTGTAATGTATGTG CTCGT-3′) and *Hin*dIII (5′-CTCCGAAGCTTAAGCC CGATATCGTCTTTCCCGTATCA-3′) restriction enzyme sequences that facilitates cloning into the $pGRE_5$-2 vector.[32]

Helper virus-free amplicon packaging

On the day before transfection, 2×10^6 BHK cells were seeded on a 60-mm culture dish and incubated overnight at 37°C. For cosmid-based packaging: the day of transfection, 250 μl Opti-MEM (Gibco-BRL, Bethesda, MD, USA), 0.4 μg of each of the five cosmid DNAs (kindly provided by Dr A Geller, Children's Hospital, Boston, MA, USA) and 0.5 μg amplicon vector DNA with or without varying amounts of pBSKS(vhs) plasmid DNA were combined in a sterile polypropylene tube.[6] For BAC-based packaging: 250 μl Opti-MEM, 3.5 μg of pBAC-V2 DNA (kindly provided by Dr C Strathdee, JP Robarts Institute, London, ON, Canada) and 0.5 μg amplicon vector DNA with or without varying amounts of pBSKS(vhs) plasmid DNA were combined in a sterile polypropylene tube.[7] The protocol for both cosmid- and BAC-based packaging was identical from the following step forward. Ten microliters of Lipofectamine Plus Reagent (Gibco-BRL) were added over a 30-s period to the DNA mix and allowed to incubate at RT for 20 min. In a separate tube, 15 μl Lipofectamine (Gibco-BRL) were mixed with 250 μl Opti-MEM. Following the 20-min incubation, the contents of the two tubes were combined over a 1-min period, and incubated for an additional 20 min at RT. During the second incubation, the medium in the seeded 60-mm dish was removed and replaced with 2 ml Opti-MEM. The transfection mix was added to the flask and allowed to incubate at 37°C for 5 h. The transfection mix was then diluted with an equal volume of DMEM plus 20% FBS, 2% penicillin/streptomycin, and 2 mM hexamethylene *bis*-acetamide (HMBA), and incubated overnight at 34°C. The following day, medium was removed and replaced with DMEM plus 10% FBS, 1% penicillin/streptomycin, and 2 mM HMBA. The packaging flask was incubated an additional 3 days and virus harvested and stored at −80°C until purification. Viral preparations were subsequently thawed, sonicated, and clarified by centrifugation (3000 *g*, 20 min). Viral samples were stored at −80°C until use. For concentrated viral stocks, viral preparations were subsequently thawed, sonicated, clarified by centrifugation, and concentrated by ultracentrifugation through a 30% sucrose cushion.[33] Viral pellets were resuspended in 100 μl PBS and stored at −80°C until use. For packaging experiments examining the effect of VP16 on amplicon titers, the cells plated for packaging were first allowed to adhere to the 60-mm culture dish for 5 h and subsequently transfected with $pGRE_5vp16$ using the Lipofectamine reagent as described above. Following a 5-h incubation, the transfection mix was removed, complete medium (DMEM plus 10% FBS, 1% penicillin/streptomycin) was added, and the cultures were incubated at 37°C until the packaging cotransfection step the subsequent day.

Viral titering

Amplicon titers were determined by counting the number of cells expressing enhanced green fluorescent protein (HSVPrPUC/CMVegfp amplicon) or β-galactosidase (HSVlac amplicon). Briefly, 10 μl of concentrated amplicon stock was incubated with confluent monolayers (2×10^5 expressing particles) of NIH 3T3 cells plated on glass coverslips. Following a 48-h incubation, cells were either fixed with 4% paraformaldehyde for 15 min at RT and mounted in Mowiol for fluorescence microscopy (eGFP visualization), or fixed with 1% glutaraldehyde and processed for X-gal histochemistry to detect the *lacZ* transgene product. Fluorescent or X-gal-stained cells were enumerated, expression titer calculated, and represented as either green-forming units per ml (gfu/ml) or blue-forming units per ml (bfu/ml), respectively.

TaqMan quantitative PCR system

To isolate total DNA for quantitation of amplicon genomes in packaged stocks, virions were lysed in 100-mM potassium phosphate pH 7.8 and 0.2% Triton X-100. Two micrograms of genomic carrier DNA was added to each sample. An equal volume of 2 × digestion buffer (0.2 M NaCl, 20 mM Tris-Cl pH 8, 50 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K) was added to the lysate and the sample was incubated at 56°C for 4 h. Samples were processed further by one phenol:chloroform, one chloroform extraction, and a final ethanol precipitation. Total DNA was quantitated and 50 ng of DNA was analyzed in a PE7700 quantitative PCR reaction using a designed *lacZ*-specific primer/probe combination multiplexed with an 18S rRNA-specific primer/probe set. The *lacZ* probe sequence was 5′-6FAM-ACCCCGTACGTCTTCCCG AGCG-TAMRA-3′; the *lacZ* sense primer sequence was 5′-GGGATCTGCCATTGTCAGACAT-3′; and the *lacZ* antisense primer sequence was 5′-TGGTGTGGGCCA-TAATTCAA-3′. The 18S rRNA probe sequence was 5′-JOE-TGCTGGCACCAGACTTGCCCTC-TAMRA-3′; the 18S sense primer sequence was 5′-CGGCTACCA-CATCCAAGGAA-3′; and the 18S antisense primer sequence was 5′-GCTGGAATTACCGCGGCT-3′.

Each 25-μl PCR sample contained 2.5 μl (50 ng) of purified DNA, 900 nM of each primer, 50 nM of each probe, and 12.5 μl of 2X Perkin-Elmer Master Mix. Following a 2-min 50°C incubation and 2-min 95°C denaturation step, the samples were subjected to 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescent intensity of each sample was detected automatically during the cycles by the Perkin-Elmer Applied Biosystem Sequence Detector 7700 machine. Each PCR run included the following: no-template control samples, positive control samples consisting of either amplicon DNA (for *lacZ*) or cellular genomic DNA (for 18S rRNA), and standard curve dilution series (for *lacZ* and 18S). Following the PCR run, 'real-time' data were analyzed using Perkin-Elmer Sequence Detector Software version 1.6.3 and the standard curves. Precise quantities of starting template were determined for each titering sample and results were expressed as numbers of vector genomes per ml of original viral stock.

Western blot analysis

BHK cell monolayers (2×10^6 cells) transfected with varying packaging components were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.5% SDS, and 50 mM Tris-Cl, pH 8). Equal amounts of protein were electrophoretically separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The resultant blot was incubated with an anti-VP16 monoclonal antibody (Chemicon, Temecula, CA, USA), and specific VP16 immunoreactive band visualized using an alkaline phosphatase-based chemiluminescent detection kit (ECL).

Cytotoxicity assays

The effect of BAC-packaged HSVlac stocks prepared in the presence or absence of VP16 and/or vhs on cell viability was determined using a lactate dehydrogenase (LDH) release-based assay (Promega, Madison, WI, USA). Equivalent expression units of virus from each packaging sample were used to transduce 5×10^3 NIH 3T3 cells in 96-well flat-bottomed culture dishes. Quantitation of LDH release was performed according to the manufacturer's instructions. Viability data were represented as normalized cell viability index.

Stereotactic injections

Mice were anesthetized with avertin at a dose of 0.6 ml per 25 g body weight. After positioning in an ASI murine stereotactic apparatus, the skull was exposed via a midline incision, and burr holes were drilled over the following coordinates (bregma, +0.5 mm; lateral −2.0 mm; and deep, −3.0 mm) to target infections to the striatum. A 33 GA steel needle was gradually advanced to the desired depth, and 3 μl (equivalent *in vitro* titer) HSVPrPUC/CMVegfp virus was infused via a microprocessor-controlled pump over 10 min (UltraMicroPump; World Precision Instruments, Sarasota Springs, FL, USA). The injector unit was mounted on a precision small animal stereotactic frame (ASI Instruments, Warren, MI, USA) micromanipulator at a 90° angle using a mount for the injector. Viral injections were performed at a constant rate of 300 nl/min. The needle was removed slowly over an additional 10-min period.

Tissue preparation and GFP visualization

Infected mice were anesthetized 4 days later, a catheter was placed into the left ventricle, and intracardiac perfusion was initiated with 10 ml of heparinized saline (5000 U/l saline) followed by 60 ml of chilled 4% PFA. Brains were extracted and postfixed for 1–2 h in 4% PFA at 4°C. Subsequently, brains were cryoprotected in a series of sucrose solutions with a final solution consisting of a 30% sucrose concentration (w/v) in PBS. Forty micron serial sections were cut on a sliding microtome (Micron/Zeiss, Thornwood, NY, USA) and stored in a cryoprotective solution (30% sucrose (w/v), 30% ethylene glycol in 0.1 M phosphate buffer (pH 7.2)) at −20°C until processed for GFP visualization. Sections were placed into Costar net wells (VWR, Springfield, NJ, USA) and incubated for 2 h in 0.1 M Tris buffered saline (TBS) (pH 7.6). Upon removal of cryoprotectant, two additional 10-min washes in 0.1 M TBS with 0.25% Triton X-100 (Sigma, St Louis, MO, USA) were performed. Sections were mounted with a fine paint brush on to subbed slides, allowed to air dry, and mounted with an aqueous mounting media, Mowiol. GFP-positive cells were visualized with a fluorescent microscope (Axioskop, Zeiss) utilizing a FITC cube (Chroma Filters, Brattleboro, VT, USA). All images used for morphological analyses were digitally acquired with a three-chip color CCD camera at 200 × magnification (DXC-9000; Sony, Montvale, NJ, USA).

Morphological analyses

Cell counts were performed on digital images acquired within 24 h of mounting. At the time of tissue processing coronal slices were stored serially in three separate compartments. All compartments were processed for cell counting and GFP(+) cell numbers reflect cell counts throughout the entire injection site. All spatial measurements were acquired using an image analysis program (Image-Pro Plus, Silver Spring, MD, USA) at a final magnification of 200 ×. Every section was analyzed using identical parameters in three different planes of focus throughout the section to prevent repeated scoring of GFP(+) cells. Each field was analyzed by a computer macro to count cells based on the following criteria: object area, image intensity (fluorescent signal) and plane of focus. Only cells in which the cell body was unequivocally GFP(+) and nucleus clearly defined were counted. Every section that contained a GFP(+) cell was counted. In addition, a watershed separation technique was applied to every plane of focus in each field to delineate overlapping cell bodies. The watershed method is an algorithm that is designed to erode objects until they disappear, then dilates them again such that they do not touch.

Statistical analyses

Statistical analyses were carried out using one-way analysis of variance (ANOVA) with plasmid construct as the between-group variable. Two-way repeated measure analyses of variance (RMANOVA) were carried out using plasmid construct as the between-group variable and time interval as a within-group variable.

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